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Three small integrin-binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases

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ABSTRACT

Matrix metalloproteinases (MMPs) are critical for development, wound healing, and for the progression of cancer. It is generally accepted that MMPs are secreted in a latent form (proMMP) and are activated only upon removal of their inhibitory propeptides. This report shows that three members of the SIBLING (Small, Integrin-Binding LIgand, N-linked Glycoprotein) family can specifically bind (K_d≈nM) and activate three different MMPs. Binding of SIBLING to their corresponding proMMPs is associated with structural changes as indicated by quenching of intrinsic tryptophan fluorescence, increased susceptibility to plasmin cleavage, and decreased inhibition by specific natural and synthetic inhibitors. Activation includes both making the proMMPs enzymatically active and the reactivation of the TIMP (tissue inhibitors of MMP) inhibited MMPs. Bone sialoprotein specifically binds proMMP-2 and active MMP-2, while osteopontin binds proMMP-3 and active MMP-3, and dentin matrix protein-1 binds proMMP-9 and active MMP-9. Both pro and active MMP-SIBLING complexes are disrupted by the abundant serum protein, complement Factor H, thereby probably limiting SIBLING-mediated activation to regions immediately adjacent to sites of secretion in vivo. These data suggest that the SIBLING family offers an alternative method of controlling the activity of at least three MMPs.

Key words: bone sialoprotein • osteopontin • dentin matrix protein 1 • matrix metalloproteinase • SIBLING

Members of the SIBLING family contain the integrin binding tripeptide, Arg-Gly-Asp (RGD) as well as several conserved phosphorylation and N-glycosylation sites (1). All of the genes cluster on human chromosome 4 and are expressed in the skeleton. There are five known members of the SIBLING family: bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) (1). Normally BSP is produced by osteoblasts, osteoclasts, osteocytes, and hypertrophic chondrocytes (2) and through its Arg-Gly-Asp sequence binds to the integrin, $\alpha_V\beta_3$. OPN (also known as Secreted Phosphoprotein 1, 2ar, and early T-lymphocyte activation 1) is expressed in multiple tissue types and can bind to $\alpha_V\beta_3$ (3–5) as well as CD44 (6,

7). DMP1 expression has been found in teeth and bone (8, 9) and can also interact with both $\alpha_V\beta_3$ and CD44 (10). OPN, BSP and DMP1 can protect cells from complement attack via initial binding to $\alpha_V\beta_3$ integrin (all three) or CD44 (OPN and DMP1) on the cell surface. The SIBLING then binds complement Factor H and the membrane-bound complex acts as a cofactor to complement Factor I thereby quenching complement-mediated cell lysis (10, 11).

Recent observations using paraffin sections have shown that BSP and OPN are expressed by multiple malignant tissues, including breast (12–14), prostate (15, 16), lung (17), and thyroid (18, 19) cancers. BSP expression is associated with poor survival in breast cancer (13) and prostate cancer (15). Similarly, OPN expression is associated with clinical severity in lung cancer (20), lymph node negative breast cancer (14), gastric cancer (6), and perhaps ovarian carcinoma (21). Recently, DMP1 has been shown to be strongly up-regulated in lung cancer (22). The neoplastic expression pattern of other SIBLING members has not been defined. Recently, OPN and/or BSP were found to be elevated in the blood of breast, lung, colon, and prostate cancer patients (23).

Matrix metalloproteinases (MMPs) are a class of hydrolytic enzymes defined by common structure and a requirement for zinc in the active site. Currently, it is accepted that the inhibitory propeptide must be removed before the MMP can be enzymatically active. After the removal of the propeptide, the MMP typically remains active until a TIMP (tissue inhibitor of matrix metalloproteinase) binds and inactivates the protease. In addition to their roles in tissue remodeling, MMPs have been postulated to play major roles in tumor cell progression and metastasis (24). In this report, we show that at least three members of the SIBLING family specifically bind to different proMMPs resulting in conformational changes that are catalytically active. Furthermore, it is shown that active MMPs inhibited by either TIMPs or low-molecular-weight synthetic inhibitors are reactivated by their corresponding SIBLING and that all these activations can be essentially reversed by complement Factor H.

METHODS

Reagents

ProMMPs, their active counterparts, TIMP1, and monoclonal antibodies that recognize both latent and active forms of MMP-2, MMP-3, and MMP-9 were obtained from Oncogene Research Products (Boston, MA) and Research Diagnostic Systems, Inc. (Minneapolis, MN). Purified human Factor H protein was obtained from Quidel Corporation (San Diego, CA). ToyoPearl activated AF-Tresyl-650 M and TSK QAE resins were obtained from TosoHaas, Inc. (Montgomeryville, PA). The MMP-2 inhibitor I, cis-9-octadecenoyl-N-hyrdoxylamide; MMP-3 Inhibitor II, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid; MMP-9 Inhibitor I, N-hydroxy-1-(4-methoxyphenyl)sufonyl-4-(4-biphenylcarbonyl)piperazine-2-carboxamide, and plasmin were obtained from Calbiochem (La Jolla, CA). The small molecular weight fluorescently quenched substrates MET-05, (7-methylcoumarin-4-yl acetic acid)-Arg-Pro-Lys-Pro-Val-Glu-Ape-Trp-Arg-Lys-(dinitrophenyl)-NH_2; MET-08, (7-methylcoumarin-4-yl acetic acid)-Arg-Pro-Lys-Pro-Tyr-Ala-Ape-Trp-Met-Lys-(dinitrophenyl)-NH_2; and MET-09, (7-methylcoumarin-4-yl acetic acid)-Pro-Leu-Gly-Leu-(diaminopropionic acid)-Ala-Arg-NH_2 were purchased from Enzyme System Products (Livermore, CA). TIMP2 was a generous gift of Dr. H. Birkedal-Hansen, NIDCR, NIH.

SIBLING production and purification

Recombinant human BSP, human OPN and bovine DMP1 with good post-translational modifications were expressed in eukaryotic cells using adenovirus constructs as described previously (10, 11). Recombinant SIBLINGs were purified from the serum-free media of the same primary cell cultures infected with their respective viruses by anion exchange chromatography under nondenaturing conditions (11). For binding studies as well as MMP activation assays, SIBLINGs were further purified by treatment with a chaotropic buffer (4 M guanidine HCl in 40 mM Tris, 1 mM DTT, pH 7.4) for 15 min at room temperature followed by dialysis and lyophilization. SIBLINGs are flexible in solution and lack sufficient cysteine residues for disulfide bond formation (1). Treatment of the SIBLINGs with denaturants (reducing agents or chaotropic agents) does not affect the "structureless" nature of SIBLINGs but will denature other proteins and enable the isolation of SIBLINGs that are free of copurifying MMPs.

SIBLING affinity chromatography

SIBLING affinity columns were made by conjugating BSP or OPN to ToyoPearl AF-Tresyl-650 M resin. 3.3 mg BSP was reacted with 0.33 g Tresyl resin in 50 mM Tris pH 8.0 containing 0.5 M NaCl overnight at 4°C. Resin was rinsed twice with 20 ml Tris buffer, and any remaining active groups were blocked by incubation for 2 h at room temperature in Tris buffer. Conjugated resin was packed into a 10 x 0.66 cm Omnifit glass column (Rainin, Woburn, MA) and equilibrated in 20 mM Tris-HCl buffer, pH 7.4 containing 10 mM $CaCl_2$, 0.02% NaN_3, 0.05% Brij 35, and 12 mM EDTA. For OPN, the affinity column consisting of 4.5 mg OPN coupled to 0.4 g of Tresyl resin following the same steps as for the BSP affinity column. Serum-free conditioned medium (B16F10 melanoma cell line) was injected onto the columns and washed in the equilibration buffer until the UV absorbance at 280 nm returned to baseline. A linear gradient to 1.0 M NaCl over 60 min (1.0 ml/min flow rate) was used to elute the bound MMP. Fractions were analyzed by zymography as below.

SDS PAGE, zymography

10% zymogram gelatin and 12% zymogram casein gels were obtained from Invitrogen, Inc. (Carlsbad, CA). Samples in zymogram gel sample buffer were electrophoresed at a constant 125 V for 90 min. Gels were processed for zymography according to the manufacturer's instructions, stained with 0.5% Coomassie Blue R 250, and bands were visualized by dynamic integrated exposure (integrating a 1/30 s exposure over 3 s) using an EagleEye II imaging system (Stratagene Corp., La Jolla, CA). For subsequent visualization of the SIBLINGs, gels were completely destained in high methanol destaining solution, rinsed in the 25% isopropanol through 4 changes over a 12 h period, and then reacted with the StainsAll solution (0.1% (w/v) containing 25% (v/v) isopropanol, 5% (v/v) formamide and 15 mM Tris-HCl, pH 8.8). 12% acrylamide gels were employed to parallel SIBLING and MMP zymogram profiles and the proteins transferred to nitrocellulose for analysis by Western blot.

Western blotting

Samples diluted in gel sample buffer were resolved by Tris/glycine SDS 12% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and transferred to nitrocellulose following standard

conditions (25). Nitrocellulose membranes were rinsed with Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-Tween). After 1 hour of incubation in blocking solution (TBS-Tween + 5% nonfat powdered milk) at room temperature, 1 μg/ml primary antibody (monoclonal anti-MMP) was incubated overnight at 4°C. The blot was washed in TBS-Tween four times for 5 min with TBS-Tween and then HRP-conjugated goat anti-mouse IgG (100 ng/ml) in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. After washing, enhanced chemiluminescence reagents were employed for signal detection (Pierce Chemical Co., Chicago, IL) with X-ray film.

Fluorescent binding studies

Intrinsic tryptophan fluorescence was monitored by excitation at 295 nm and emission from 300 to 500 nm using a Photon Technology International Series M fluorimeter. The relative change in fluorescence in the area under the emission curve was used to determine binding curves by calculating fractional acceptor saturation vs. nM SIBLING added. Fractional acceptor saturation (f_a) was determined by calculating $f_a = (y - y_f)/(y_b - y_f)$, where y_f and y_b are the area under the curve of the fluorescent emission profile of free and fully bound MMP, respectively. Scatchard plots were made by fitting the transformed data to the function $r/[C_S] = n/K_d - r/K_d$, where r represents the binding function, C_S represents total ligand concentration, n represents the number of binding sites, and K_d represents the dissociation constant. The initial latent or active MMP concentration was 3.5 nM and concentrated SIBLINGs were added in nM amounts. Both latent and active MMPs, as well as SIBLINGs were dissolved in Hank's balanced salt solution. To determine stoichiometry, a titration was carried out under conditions with an excess of MMP (10-fold higher than the dissociation constant). The stoichiometry was ascertained in each case by transforming the data into a plot of fractional saturation vs. the ratio of total ligand C_S to total acceptor C_A. Extrapolating the two linear segments of the graph to their point of intersection defined the MMP and SIBLING stoichiometry.

High-molecular-weight substrates

Fluorescein-conjugated gelatin (Molecular Probes, Inc., Eugene, OR) or casein (Pierce Chemical Co., Chicago, IL) substrates were used to follow proteolytic activity on more natural macromolecular substrates. These substrates are so highly substituted with fluorescein moieties that the fluorescent signal is self-quenched until proteolytic cleavage liberated fragments, and a robust fluorescent emission is measured. 1.4 nM MMP (latent or active MMP-2, MMP-3, and MMP-9) was reacted with increasing concentrations of SIBLINGs or buffer alone and 12.5 μg/ml of the fluorescein-substrate conjugate in 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$. Fluorescent data were acquired with excitation at 485 nm and emission at 535 nm. In indicated experiments, the general MMP inhibitor 1,10 phenanthroline was added. A final 1,10 phenanthroline concentration of 1 mM in zymograms and 1.4 μM in enzyme assays were made by the appropriate dilution from a 100 mM stock solution dissolved in DMSO. In experiments with TIMPs, the inhibitors were preincubated in equimolar amounts with the MMPs before the addition of the SIBLINGs. Factor H was added at levels equimolar to the SIBLINGs.

Low-molecular-weight substrates

The activities of latent and active MMPs in the presence and absence of SIBLINGs and after plasmin treatment were measured using the small fluorescence-quenched substrates (26). These

substrates have two fluorescent moieties covalently attached usually at either end of the peptide such that the fluorescent signal of the intact substrate is effectively quenched. Cleavage of the peptide results in loss of quenching and an enhanced fluorescence signal. The peptide substrates were initially dissolved in DMSO and each assay carried out at 25°C in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35 and 1% DMSO (v/v). ProMMPs (140 nM) were added to 400 nM substrate in the presence or absence of SIBLING (140 nM). Substrate cleavage was monitored using a Perkin Elmer Victor 2 multilabel plate reader with 330 excitation and 390 emission filters.

Plasmin susceptibility

SIBLING and proMMP concentrations were 140 nM, plasmin 20 nM in 50 mM HEPES, pH 7.5, 150 mM NaCl, 8 mM $CaCl_2$, 0.01% Brij 35, and 1% DMSO. Low-molecular-weight fluorescently quenched methylcoumarin-derivatized substrates were employed at a concentration of 400 nM to follow MMP activity in the presence and absence of SIBLINGs and plasmin. Plasmin itself will not digest the peptide substrate under these conditions.

RESULTS

SIBLINGs and MMPs copurify

Full-length BSP, OPN, and DMP1 cDNA were subcloned into adenovirus vectors and expressed in human bone marrow stromal cells. Each SIBLING was purified from the serum-free media of the same primary cell cultures infected with their respective viruses to ≥ 95% purity by anion exchange chromatography under nondenaturing conditions (11). Interestingly, when purity was further assessed using casein zymography, each HPLC-purified SIBLING exhibited a single but different band of proteolytic activity (Fig. 1*A* and *B*). For DMP1 (M_r of 110–120 kDa on PAGE), a zymogram-positive band was seen at ~84 kDa, consistent with MMP-9. BSP (M_r of 70–80 kDa) had an associated zymogram-positive band of ~66 kDa, consistent with MMP-2. Finally, OPN (M_r of 50– 60 kDa) had an associated zymogram positive band of ~45 kDa consistent with MMP-3. Bands originally visible on the zymogram did not appear in gels treated with 1,10-phenanthroline, showing that the copurifying proteolytic activity arose from metalloproteinases (data not shown). This copurification of MMP-2 with BSP, MMP-3 with OPN, and MMP-9 with DMP1 was verified by electrophoresing the same preparations of SIBLINGs on SDS polyacrylamide gels followed by blotting to nitrocellulose and probing with monoclonal antibodies against the three MMPs (Fig. 1*C*, *D*, and *E*).

SIBLING and MMP affinity purification

The specificity observed in copurification of individual MMPs with recombinant SIBLINGs was confirmed in two cases by showing that SIBLINGs could be used to affinity purify their respective MMPs from conditioned media. BSP and OPN were conjugated to resins and serum-free conditioned B16F10 medium was used as a source of MMPs, including MMP-2, MMP-3, and MMP-9. Elution of bound material in a salt gradient yielded multiple peaks as monitored at A 280 m (Fig. 2*A* and *B*). Aliquots of fractions (denoted by arrows) were analyzed by zymography. Zymogram bands with a M_r corresponding to latent and active MMP-2 (for the BSP affinity column) and proMMP-3 (for the OPN affinity column) and were visible only in peaks eluted at higher (~0.3 M) salt (Fig. 2*C* and *D*). The gradient-eluting bands did not appear

in zymogram gels treated with 1,10-phenanthroline. The same fractions were also analyzed by Western blot and immunoreactive MMP-2 (from the BSP affinity column) and immunoreactive MMP-3 (from the OPN affinity column) were identified (Fig. 2E and F). A DMP1 affinity column was not made due to insufficient amounts of highly purified protein.

SIBLING and MMP binding specificity

Copurification by virtually identical methods of a single but different MMP with each SIBLING suggested that there were specific and relatively strong interactions occurring between the proteins. The relative abundance of tryptophan residues in the MMPs was exploited by carrying out intrinsic fluorescence studies of purified, authentic latent and active MMP protein binding to SIBLINGs. ProMMP-2 contains 15 tryptophan residues, proMMP-3 contains 8, and proMMP-9 contains 13. In contrast, mature OPN and DMP1 each contain 1 tryptophan residue whereas BSP contains none. Intrinsic tryptophan fluorescence measurements of macromolecules yield information about conformation, binding, and solvent interactions. Titration of proMMP-2 with BSP yielded a proportional amount of quenching of the MMP's tryptophan emission spectra (Fig. 3*A*). Titration of proMMP-3 with OPN and proMMP-9 with DMP1 also yielded a similar quenching of the MMPs' tryptophan emission spectra (Fig. 3*B* and *C*). Quenching of the MMP tryptophan fluorescent signal is consistent with a significant conformational change (exposing internal tryptophan residues to a more polar environment) as a direct result of the binding of its corresponding SIBLING. The binding of each SIBLING to its preferred proMMP was saturable (Fig. 3*D*, *E*, and *F*). When the interaction of SIBLINGs with propeptide-free (active) MMPs was studied by intrinsic fluorescence, saturable binding was again observed only for the specific pairs; BSP and active MMP-2, OPN and active MMP-3, and DMP1 and MMP-9 (Fig. 3*D*, *E*, and *F*). K_d values of 2.9 ± 0.9 and 0.3 ± 0.1 nM for BSP binding to latent and active MMP-2; 0.55 ± 0.01 and 0.17 ± 0.01 nM for OPN binding to latent and active MMP-3, and 0.41 ± 0.04 and 0.77 ± 0.01 nM for DMP1 binding to latent and active MMP-9 were determined from Scatchard plots (Fig. 3*G*, *H*, and *I*). The stoichiometry of binding between SIBLINGs and their respective proMMPs was found to be 1:1 for all three combinations (Fig. 3*J*, *K*, and *L*). All combinations of mismatches of SIBLINGs and both pro and active MMPs (ex. BSP with proMMP-3 or DMP1 with MMP-2) yielded either no binding curve at all or no saturation, thereby again showing specificity of the SIBLING to MMP interactions (data not shown).

SIBLING–MMP complexes modify the protease activity

The fluorescence spectroscopy observations suggesting that SIBLINGs induce conformational changes in their corresponding MMP partners led to an investigation of whether SIBLING binding affected MMP structure and function. First, samples of correctly matched proMMPs and SIBLINGs were incubated for 1 h at room temperature and resolved by casein and gelatin zymography to monitor propeptide presence. The addition of SIBLING to proMMP did not appear to cause significant cleavage (auto-activation) to the lower molecular weight, active form (Fig. 4).

Next the SIBLING–proMMP complex was tested for susceptibility of the proMMP to removal of its propeptide by an added protease. Plasmin is normally an inefficient activator of MMP-2 and MMP–9 (27) and treatment of proMMP-2 or proMMP-9 with plasmin resulted in minimal generation of zymogram bands corresponding to the "active," that is, lower M_r forms (Fig. 5*A*). The addition of BSP to proMMP-2 and DMP1 to proMMP-9 and subsequent incubation with

plasmin, however, did result in greater levels of the small, active form of the MMP as measured by zymography. As controls, neither the SIBLINGs alone, nor SIBLINGs incubated with plasmin led to the appearance of zymogram bands corresponding to active MMPs.

An increased susceptibility of SIBLING–proMMP complexes to cleavage and activation by plasmin was also seen when enzyme activity was monitored by incubation with the MMP-specific, small, fluorescent substrates. Plasmin activation of proMMP-2 and proMMP–9 was increased by treatment with SIBLING (BSP and DMP1, respectively). Surprisingly, treatment of proMMP with SIBLING alone also led to a two- to threefold increase in proteolytic activity, as measured using the low-molecular-weight substrates. Neither BSP nor DMP1 alone exhibited any activity, demonstrating that the purification procedure for SIBLINGs removed residual MMPs that had initially copurified with them, (Fig. 5*B*). Taken together, these data are consistent with SIBLING binding altering proMMP structure. The SIBLING–proMMP complex displays catalytic activity on small substrates and the propeptide is more readily cleaved by an added protease than is the proMMP alone.

SIBLINGs and proMMP activation

To measure potential enzymatic effects of the binding of the SIBLINGs to proMMPs, a fluorescent substrate (casein- or gelatin-fluorescein conjugate) assay was employed to screen the protease activity of the proMMPs. ProMMP-2, proMMP-3, and proMMP-9 were titrated with increasing concentrations of all three SIBLINGs or with vehicle alone and the resulting enzyme activity measured by increased fluorescence signal. Increased proteolytic activity was observed only for the three strong proMMP-SIBLING binding pairs and a graded dose–response was evident (Fig. 6*A*, *B*, and *C*). When the strong binding SIBLING was added to its corresponding active MMP, enzymatic activity was not significantly changed (Fig. 6*D*, *E*, and *F*). This suggests that the binding of the SIBLING with its active MMP partner did not interfere with its normal proteolytic activity.

The properly matched SIBLING–proMMP pairs showed a dose–response increase in the amount of gelatin (proMMP-2 and proMMP-9) or casein (proMMP-3) digested (Fig. 6*G*, *H*, and *I*). Incubation of SIBLING with substrate was no different from substrate alone, showing that the increase in activity in the proMMP + SIBLING was not caused by any proteolytic activity in the SIBLING preparations used for these experiments. Mismatched pairs of SIBLINGs and proMMPs (BSP with proMMP-3 and proMMP–9, OPN with proMMP-2 and proMMP–9, and DMP1 with proMMP-2 and proMMP-3) analyzed under identical conditions each yielded an activity (as measured by relative change in fluorescence over time) equivalent to that of the residual activity of each commercial proMMPs alone (Fig. 6*J*, *K*, and *L*). Given that there was no observed increase in the amount of propeptide-free enzyme in all of these SIBLING-proMMP pairs (see above), it is reasonable to hypothesize that the increase in activity is due to a conformational change in the protease that allows its propeptide to be removed from the active site and thereby permit the digestion of both small and large macromolecular substrates.

SIBLINGs restore activity to inhibited MMPs

The quenching of tryptophan fluorescence and the increase in activity caused by SIBLING binding to the proMMP are consistent with an alteration in the local structure near the active site. The addition of BSP increased the activity of proMMP-2 more than twofold, whereas addition of

the specific inhibitor caused a reduction in the basal activity (Fig. 7*A*). The activity of the proMMP-2 + BSP complex treated with equimolar amounts of the inhibitor was 84% of that shown by the SIBLING + proMMP complex alone and sixfold higher then that of proMMP + inhibitor. That the increase in proMMP activity did not arise from activity copurifying with BSP was indicated by the lack of additional activity seen upon addition of BSP alone to the assay. Similarly, the abilities of the proMMP-3+OPN complex and the proMMP-9+DMP1 complex to be inhibited by equimolar amounts of their specific MMP inhibitors resulted in only a 20–25% reduction in the maximum SIBLING-stimulated rate (Fig. 7*D* and *G*). As was the case for BSP, the relative rate of change in fluorescence was not significantly different between proMMP, SIBLING, or inhibitor controls. In contrast to the specific inhibitors, 1,10 phenanthroline (which disrupts MMP activity by chelating and removing the active site required zinc ion) blocked proMMP activity even in the presence of SIBLING.

The active forms of the MMPs also exhibited quenching of tryptophan fluorescence emission upon binding their specific SIBLING partner. The possibility that SIBLING binding also altered inhibitor interaction with active MMPs was investigated. Specific low-molecular-weight inhibitors were again used to block active MMP activity on the fluorescein-labeled substrate in the presence of SIBLINGs. MMPs were incubated with either 1) vehicle, 2) equimolar SIBLING, 3) equimolar MMP-specific inhibitor, 4) MMP-specific inhibitor and SIBLING, or 5) SIBLING + 1,10 phenanthroline. The addition of SIBLING caused no significant change in MMP activity, whereas the inhibitor-treated MMP exhibited an expected dramatic loss of activity (Fig. 7*B*, *E*, and *H*). The addition of the corresponding SIBLING, however, rescued much of the original activity, even in the presence of equimolar amounts of specific inhibitor. As was the case for proMMPs, SIBLINGs were not able to restore activity to active MMPs treated with 1,10 phenanthroline.

The effect of SIBLING on the ability of the specific inhibitors to decrease MMP enzyme activity was further investigated by studying the dose–response. The activity of authentic active MMPs + equimolar SIBLINGs were measured using the fluorescein-gelatin or casein fluorescent assay in the presence of increasing concentrations of the MMP-specific inhibitors. SIBLINGs did not alter active MMP activity, whereas the addition of 1.6 nM (equimolar) MMP-specific inhibitor to MMP alone decreased activity dramatically. When the complex of equimolar active MMP + SIBLING was treated with increasing concentrations of the inhibitor, significant loss of activity was observed but only at substantially higher concentrations (Fig. 7*C*, *F*, and *I*).

Because MMPs occur in vivo associated with TIMPs, the effect of SIBLINGs on the activity of MMP + TIMP complexes was investigated. Treatment of active MMP-2, MMP-3, or MMP–9 with equimolar amounts of the appropriate TIMP reduced the enzymatic activity of the MMP between 85 and 98% (Fig. 8). The addition of the correctly matched SIBLING to the TIMP-inhibited MMP caused a restoration of much of the original proteolytic activity. The addition of BSP to TIMP2-inhibited MMP-2 caused a fivefold increase in enzyme activity over that of the TIMP-MMP-2 complex alone. Similarly, the presence of equimolar OPN lead to a sevenfold increase in activity of TIMP1-inhibited MMP-3, whereas DMP1 restored TIMP1-inhibited MMP-9 activity over tenfold. It is a reasonable hypothesis that the conformational change in the active MMP upon binding its SIBLING partner lowers the affinity of the TIMP (and low-molecular-weight inhibitors) for the active site of the MMP thereby enabling substrate access.

Reversal of SIBLING-induced activity by Factor H

BSP, OPN and DMP1 have previously been shown to bind to Factor H with high affinity, 10–100 fold higher than that just described for their partner MMPs (10, 11). This binding has been shown in another context to be sufficiently strong to completely mask serum BSP and OPN in standard ELISA assays (23). The question arises as to whether Factor H can compete with MMPs for SIBLING binding and thereby affect each SIBLING's interactions with its respective proMMP and active MMPs. The gelatin and casein fluorescein conjugate assays were used to investigate whether TIMP-inhibited MMPs, which had regained enzymatic activity by the addition of their corresponding SIBLING could then have their enzymatic activity altered by the subsequent addition of purified Factor H. Addition of equimolar Factor H to samples containing SIBLINGs, activated MMPs and TIMPs caused a 75% reduction in the BSP-induced recovered activity for MMP-2, a 40% reduction for MMP-3 and a 90% reduction for MMP-9 (Fig. 8*A*, *B*, and *C*). The higher affinity of Factor H for the SIBLING protein appears to promote the removal of the SIBLING from the SIBLING-MMP complex thereby permitting the MMP to reverse its conformation and allow the TIMP to again bind to the active site and reinhibit the enzyme.

The action of Factor H on the SIBLING-mediated activation of proMMPs was also investigated. Reaction mixtures consisting of 1) proMMP alone, 2) proMMP + SIBLING, or 3) proMMP + SIBLING +Factor H were incubated with the fluorescein-gelatin conjugate and the fluorescent signal recorded for up to 8 h. The addition of Factor H caused the rate of substrate digestion by the SIBLING-activated proMMP complex to decrease suggesting that the removal of the SIBLING from the proMMP resulted in the reinactivation of the catalytic activity by the still-attached propeptide (Fig. 8*D*, *E*, and *F*). The Factor H-mediated decrease in activity was observed for all three SIBLING-proMMP pairs consistent with the higher affinity Factor H binding and removing the SIBLING from the SIBLING-proMMP complexes. The addition of Factor H alone to the proMMPs or active MMPs had no effect on the observed activity (data not shown). These results support the hypothesis that the propeptide is not removed in order to create the enzymatic activity in the proMMP-SIBLING pairs.

DISCUSSION

In this study, we have demonstrated that three members of the SIBLING family (BSP, DMP1, and OPN) can each bind to different latent and the corresponding active MMPs (MMP-2, MMP-9, and MMP-3, respectively) in a 1:1 stoichiometry with nM affinity in vitro. This has been demonstrated by 1) copurification of the pairs through nondenaturing chromatographic columns; 2) solution phase intrinsic fluorescence binding studies; and 3) showing that BSP and OPN affinity columns can be used to purify MMP-2 and MMP-3, respectively, from media containing several MMPs. In addition, we have shown that purified SIBLINGs added to their respective authentic proMMPs cause an increase in proteolytic activity without removal of the inhibitory propeptide. In all of these observations, SIBLINGs and proMMPs showed specificity in their partnering, with BSP binding to and activating proMMP-2, OPN activating proMMP-3 and DMP1 activating proMMP-9. Other pairings of the SIBLINGs and proMMPs did not lead to binding saturation or to significant increases in proteolytic activity when studied in equimolar stoichiometries. Binding of the same preferred pairs of proteins was also seen for the active forms of the MMPs, although no changes in proteolytic activity against natural substrates were observed.

We have observed 1) fluorescent quenching during SIBLING binding titration (indicating a change in the microenvironment of the MMP's tryptophans); 2) increased susceptibility of proMMP-SIBLING pairs to plasmin digestion; 3) increased enzymatic activity of the proMMPs upon binding their specific SIBLING partner; 4) reduced ability of specific low-molecular-weight inhibitors to block SIBLING + MMP complexes; and 5) restoration of activity to TIMP-inhibited MMPs by the corresponding SIBLING. These observations are consistent with binding of SIBLING to MMP being associated with structural changes in the MMP. Our hypothesis is that these conformational changes induced in the structure of the MMP by the binding of the SIBLING partner can at least partially disrupt interaction of inhibitors with enzyme's active site, presumably by lowering the affinity of the tested inhibitors for their MMPs. In vivo, this result is likely to mean that even in the presence of TIMPs, MMPs may be enzymatically active in regions of locally high concentrations of specific SIBLINGs.

Complement Factor H, a high-abundance serum protein known to have a high affinity for these three SIBLINGs, was shown to reverse the SIBLING-induced resistance of the active MMP to TIMPs, presumably by binding and removing the SIBLING partner from the complex. The decrease by Factor H of SIBLING-mediated proMMP activation is also consistent with competition between the MMP and Factor H for the binding of the SIBLING. That a portion of the activity of the three proMMP–SIBLING pairs returned to their normal low levels upon the addition of Factor H would suggest that the propeptide can reinsert into the active site and return the protease to its inactive state. However, that some enzymatic activity remains after the addition of the Factor H also suggests that the propeptide reinsertion is not always successful under these specific in vitro conditions. Factor H invariably prevents the active complexes from forming when it binds to the SIBLINGs before they can complex with their partnering MMPs. Our previous work had indicated that these three SIBLING family members in solution were rapidly bound by complement Factor H. This suggests that the activation of the proMMP or reactivation of TIMP-inhibited MMPs by the simple binding of their respective SIBLINGs will be limited to short distances from their sites of secretion due to the abundance of Factor H in the body.

In vivo, the proforms of the MMPs are generally fully activated through proteolytic cleavage of the inhibitory propeptide. Interestingly, TIMP-2, a protein that can inhibit the catalytic activity of MMP-2, is also thought to play a role in at least one mechanism of activation of propeptide cleavage (28). This activation of proMMP-2 has been proposed to involve formation of a complex of the proMMP-2 with TIMP-2 and MT1-MMP (MMP-14) on the cell surface. A second, TIMP-2-free, MT1-MMP then activates the MMP-2 by cleaving off the propeptide. By analogy, it is interesting to hypothesize that proMMPs undergoing a conformational change when binding their SIBLING partners may become better substrates for other proteases in vivo that result in the removal of the inhibitory propeptides. This paper has shown such an increase in susceptibility to at least one protease, plasmin.

Finally, because the expression of two of these SIBLINGs—BSP and OPN—have been correlated with cancer progression and severity, it is interesting to consider that these proteins may be locally activating their corresponding proteases in vivo. All three MMPs have been shown to be important in tumor development and metastasis (24). The modulation of MMP activity by SIBLINGs may even include SIBLINGs coming from the tumor cells and the MMPs from the nearby stroma. Furthermore, because MMPs have been shown to be associated with vascular invasion (29, 30) and that BSP and OPN have been shown to possess angiogenesis

activity in vivo (31, 32), it may be that the two observations can be linked by the mechanism of MMP activation presented in this report. By defining the basic biochemistry of these interactions, we now possess the requisite structural knowledge and analytical tools to pursue meaningful tissue culture and in vivo studies.

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Fig. 1

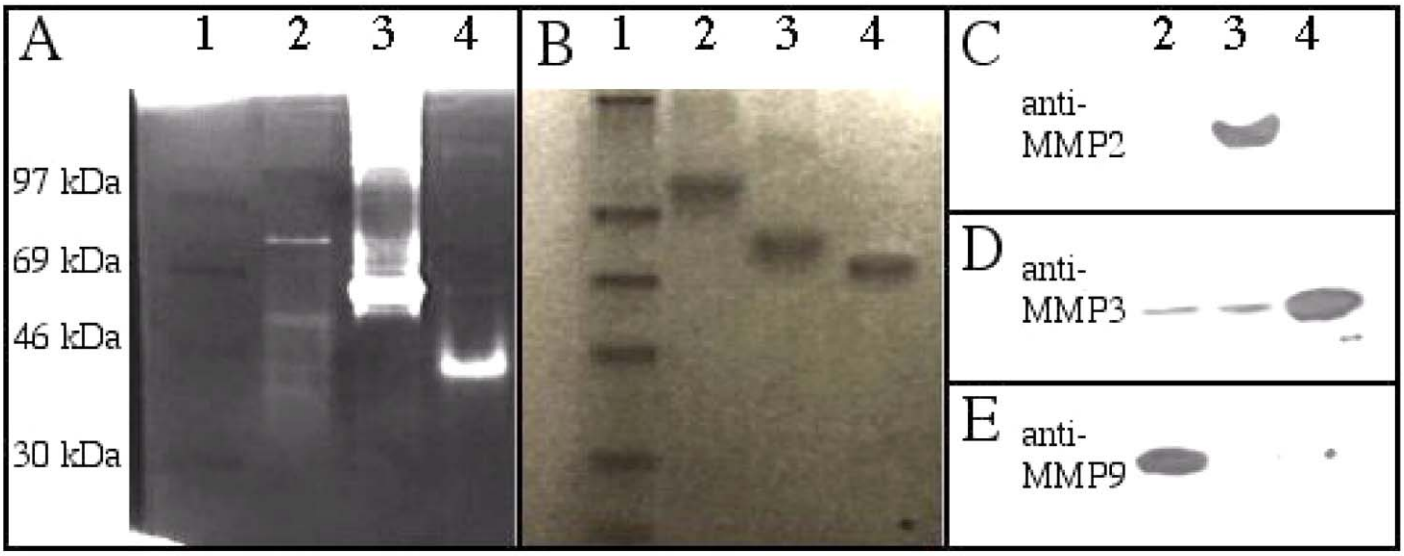

Figure 1. MMPs copurify with SIBLINGs. Recombinant proteins were purified from essentially identical culture media sources under nondenaturing conditions as described (11). Ten micrograms of each SIBLING were run on a casein zymogram gel. After electrophoresis, the gel was processed for zymography. (***A***) The gel was stained with Coomassie Blue and visualized by dynamic integrated exposure. (***B***) The destained gel was restained with StainsAll to visualize the acidic SIBLINGs. Lane 1, molecular weight standards; lane 2, DMP1; lane 3, BSP; lane 4, OPN. A 12% acrylamide gel was loaded with the same samples, electrophoresed, transferred to nitrocellulose membranes, probed with anti-MMP-2 (***C***), anti-MMP-3 (***D***) or anti-MMP-9 (***E***) and detected by chemiluminescence.

Fig. 2

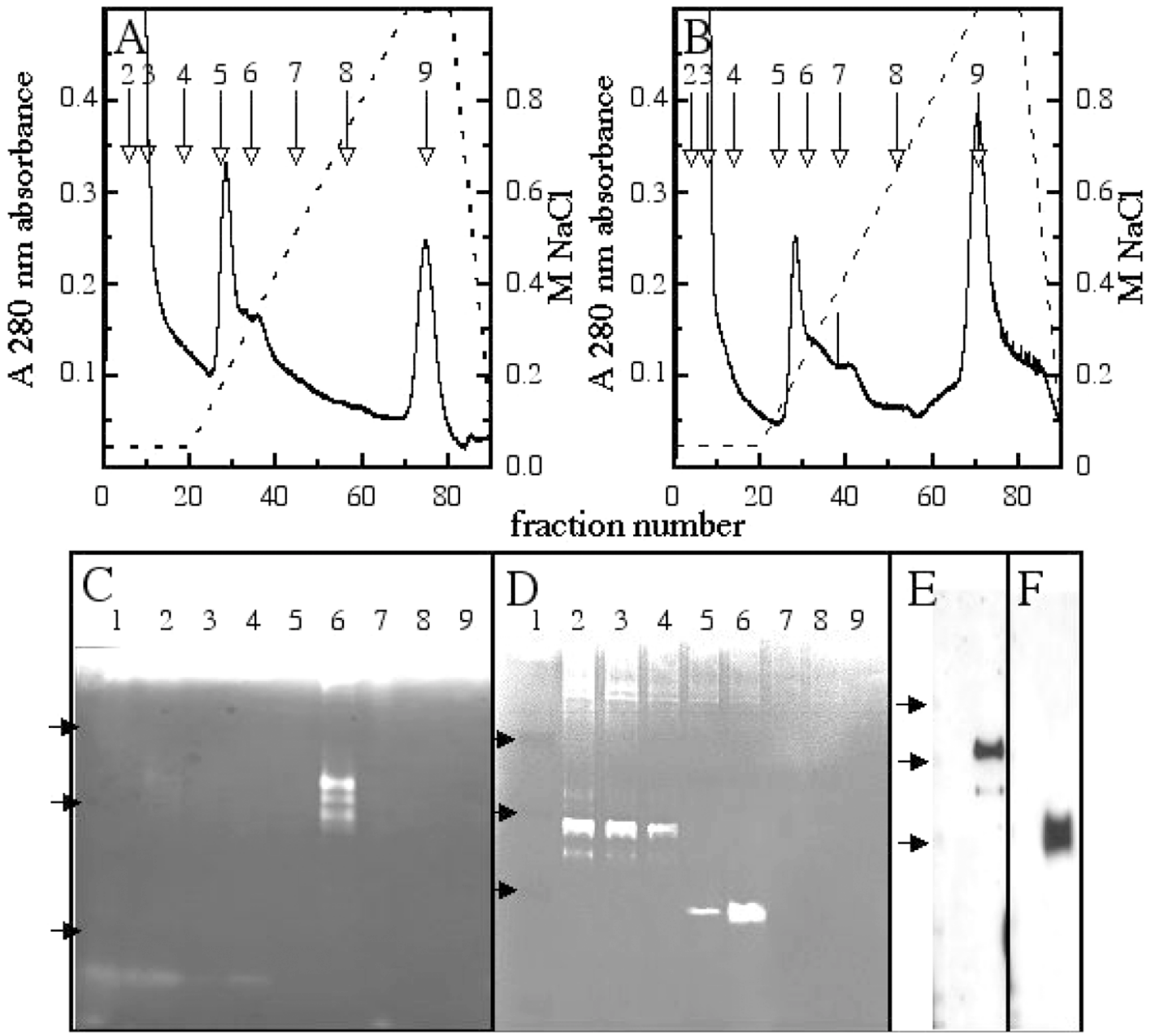

Figure 2. Affinity purification of specific MMPs on SIBLING columns. Serum-free conditioned medium from B16F10 cells was loaded onto the BSP-affinity column (***A***) or OPN-affinity column (***B***) and washed until the absorbance at 280 nm returned to background. A linear salt gradient was used to elute bound material. Fractions corresponding to numbered arrows were analyzed by gelatin (***C***) or casein (***D***) zymography. Lane 1, molecular weight standards; lanes 2 through 9 correspond to fraction numbers in panels A and B. The UV-absorbing peak at 1 M salt is an apparent buffer absorbance artifact, as no protein was evident on SDS PAGE (lane 9). 12% acrylamide gel loaded with aliquots of fraction 6 from each affinity column, electrophoresed and transferred to nitrocellulose membranes. Fraction 6 from the BSP affinity column was probed with anti-MMP-2 (***E***), while fraction 6 from the OPN affinity column was probed with anti-MMP-3 (***F***), and the immunoreactive material detected by chemiluminescence. The migration positions of the 97, 69, and 46 kDa protein standards are marked by the arrows.

Fig. 3

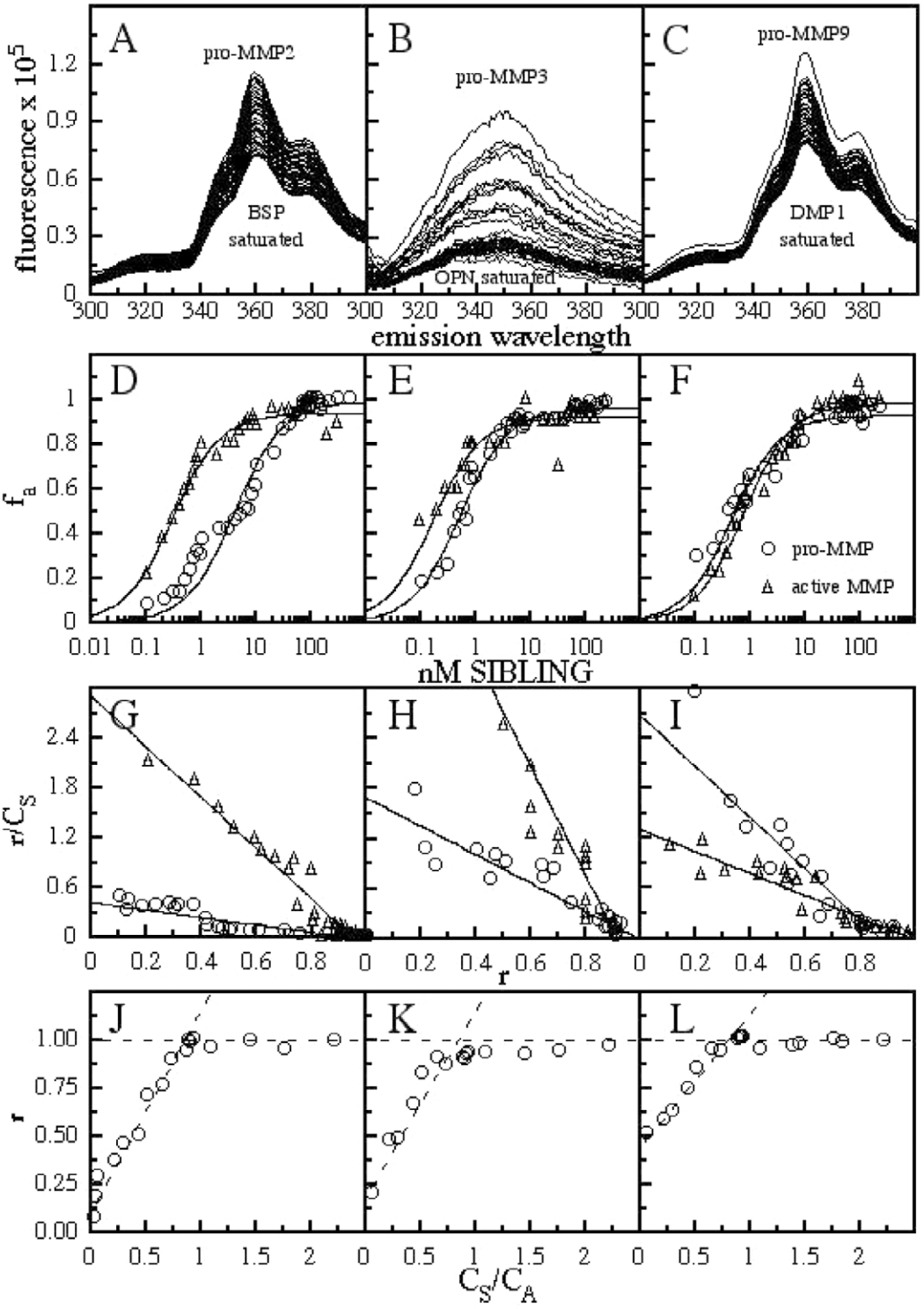

Figure 3. SIBLING binding to MMPs by intrinsic fluorescence titration. Binding interactions were investigated by titrating 3.5 nM (***A***) proMMP-2, (***B***) proMMP-3, and (***C***) proMMP-9 with BSP, OPN, or DMP1, respectively. The areas under the emission peak curves were used to determine binding functions by calculating fractional acceptor saturation of pro- (O) or active (Δ) MMP-2 with BSP (***D***), pro- (O) or active (Δ) MMP-3 with OPN (***E***), and pro- (O) or active (Δ) MMP-9 with DMP1 (***F***). Scatchard plots of proMMP-2 and active MMP-2 with BSP (***G***), proMMP-3 and active MMP-3 with OPN (***H***), as well as proMMP-9 and active MMP-9 with DMP1 (***I***) were determined. The stoichiometry was defined for BSP and proMMP-2 (***J***), OPN and proMMP-3 (***K***), and DMP1 and proMMP-9 (***L***) as described in Experimental Procedures.

Fig. 4

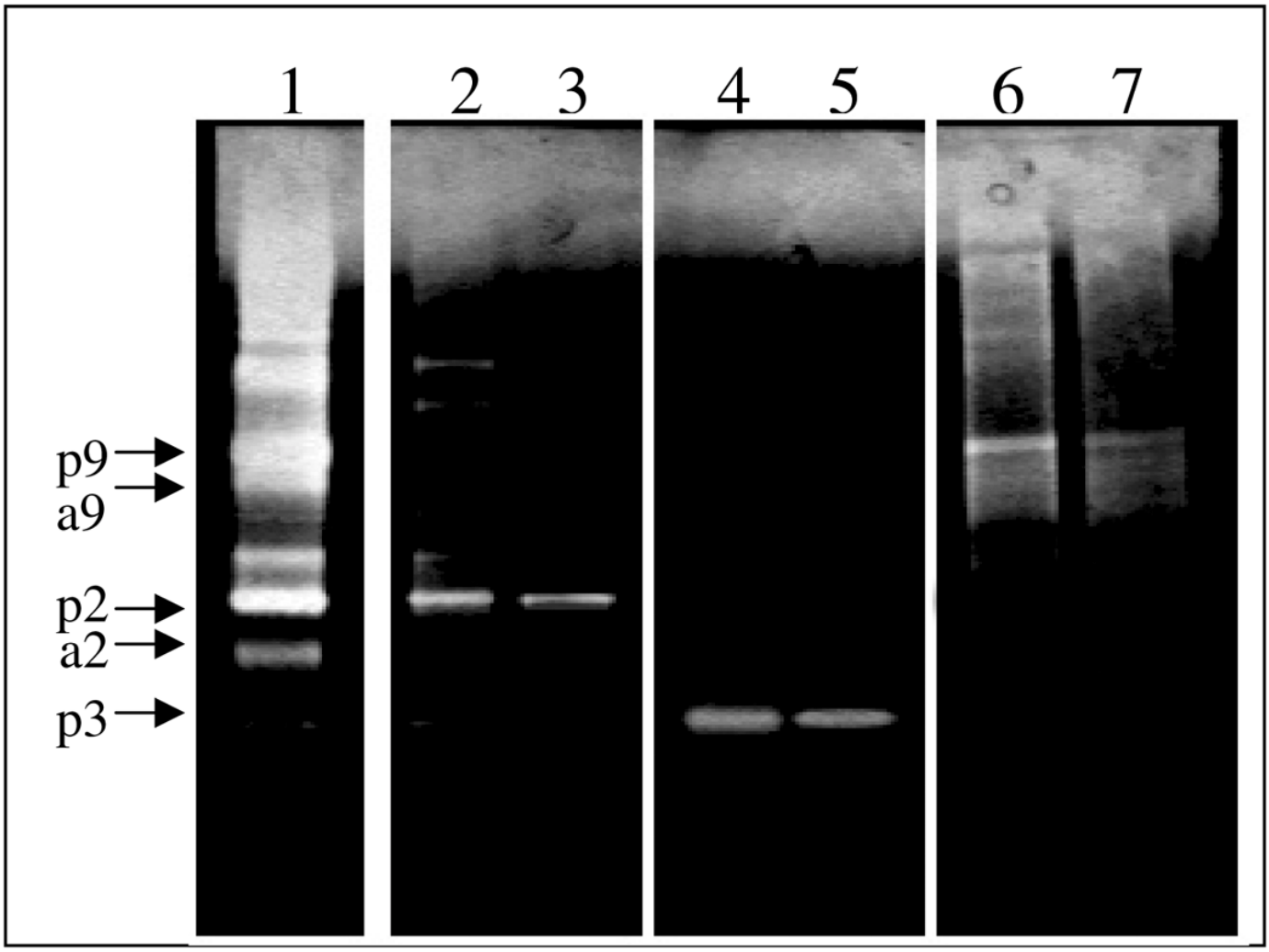

Figure 4. SIBLINGs bind but do not result in significant auto-cleavage of the MMP propeptides. Equivalent nanomolar quantities (140 nM) of correctly matched pro-MMPs and SIBLINGs were incubated for 1 h and resolved by zymography to monitor propeptide presence and relative zymogen levels. Pro MMP-2 and proMMP-9 samples were resolved by gelatin zymography (lanes 1, 2, 3, 6, and 7) while proMMP-3 containing samples were resolved by casein zymography (lanes 4 and 5). Lane 1, MMP standards; lane 2, proMMP-2; lane 3, proMMP-2 + BSP; lane 4, proMMP-3; lane 5, proMMP-3 + OPN; lane 6, proMMP-9; lane 7, proMMP-9 + DMP1. Abbreviations, P2, proMMP-2; a2, active MMP2; p3, proMMP-3; a3, active MMP-3; p9, pro-MMP-9, a9; active MMP9.

Fig. 5

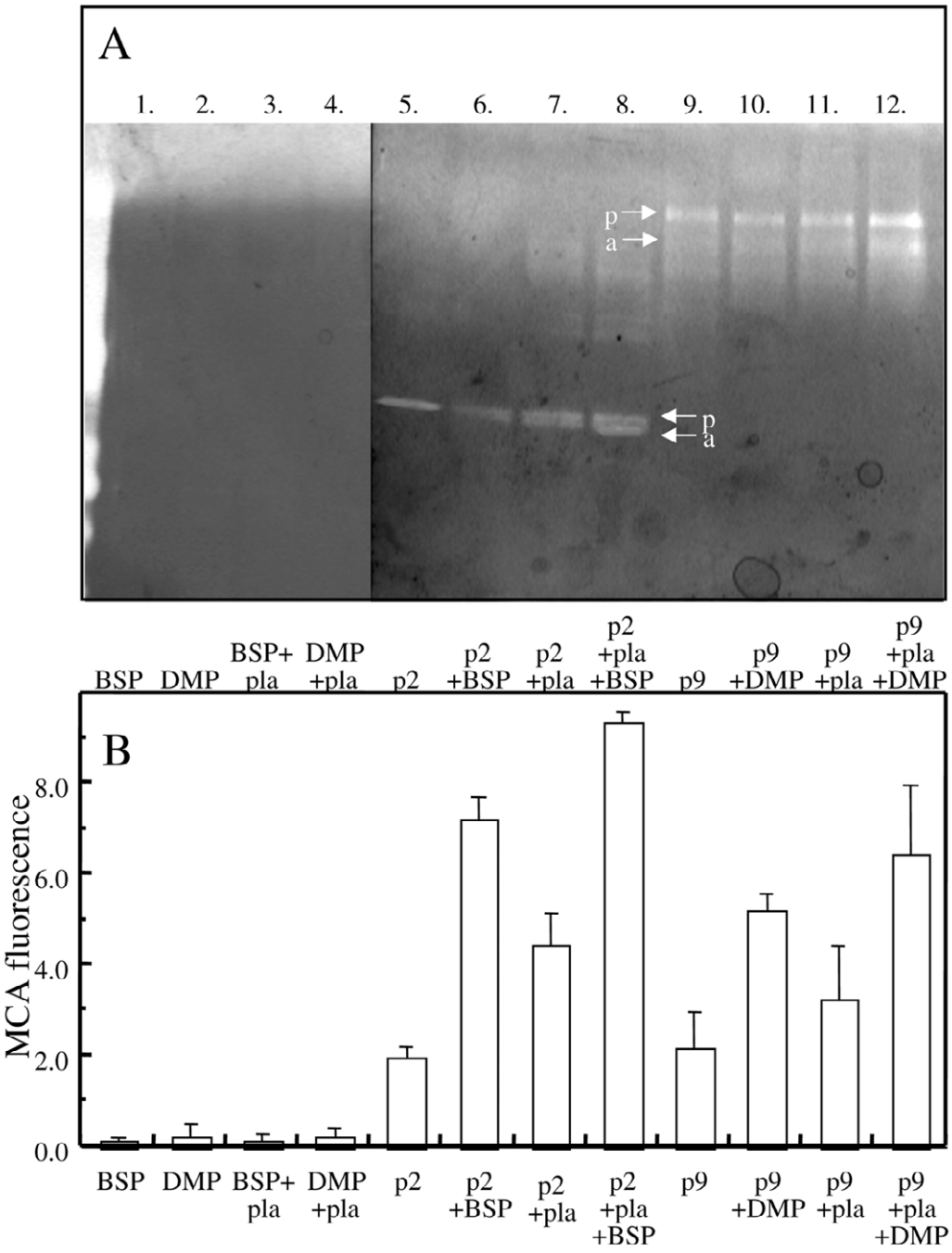

Figure 5. SIBLING–MMP complexes have altered plasmin susceptibility. Changes to the structure of MMPs caused by SIBLING binding were studied by determining the enzyme susceptibility of the MMP–SIBLING complex to digestion with plasmin. As negative controls, BSP (lane 1), DMP1 (lane 2), BSP + plasmin (lane 3) and DMP1 + plasmin were incubated for 15 min at 37 C and analyzed by zymography. In addition, latent 140 nM MMP-2 was incubated alone (lane 5), with equimolar BSP (lane 6), with plasmin (lane 7) or with equimolar BSP followed by plasmin (lane 8), while 140 nM proMMP-9 was incubated either alone (lane 9), with equimolar DMP1 (lane 10), with plasmin (lane 11) or with equimolar DMP1 followed by plasmin (lane 12) for 15 min. The samples were resolved by zymography (***A***). Note the appearance of significant amounts of a slightly lower band ("active" MMP) only in the plasmin-treated samples containing the SIBLING partners. Abbreviations, p2, proMMP-2; p9. ProMMP-9; pla, plasmin. Arrowheads indicate migration position of proMMP (p) and MMP (a) The enzymatic activities of the same 12 conditions were also determined after 15 min incubation by measuring the relative fluorescent yield from small molecular weight MMP-specific substrates as described in Experimental Procedures (***B***).

Fig. 6

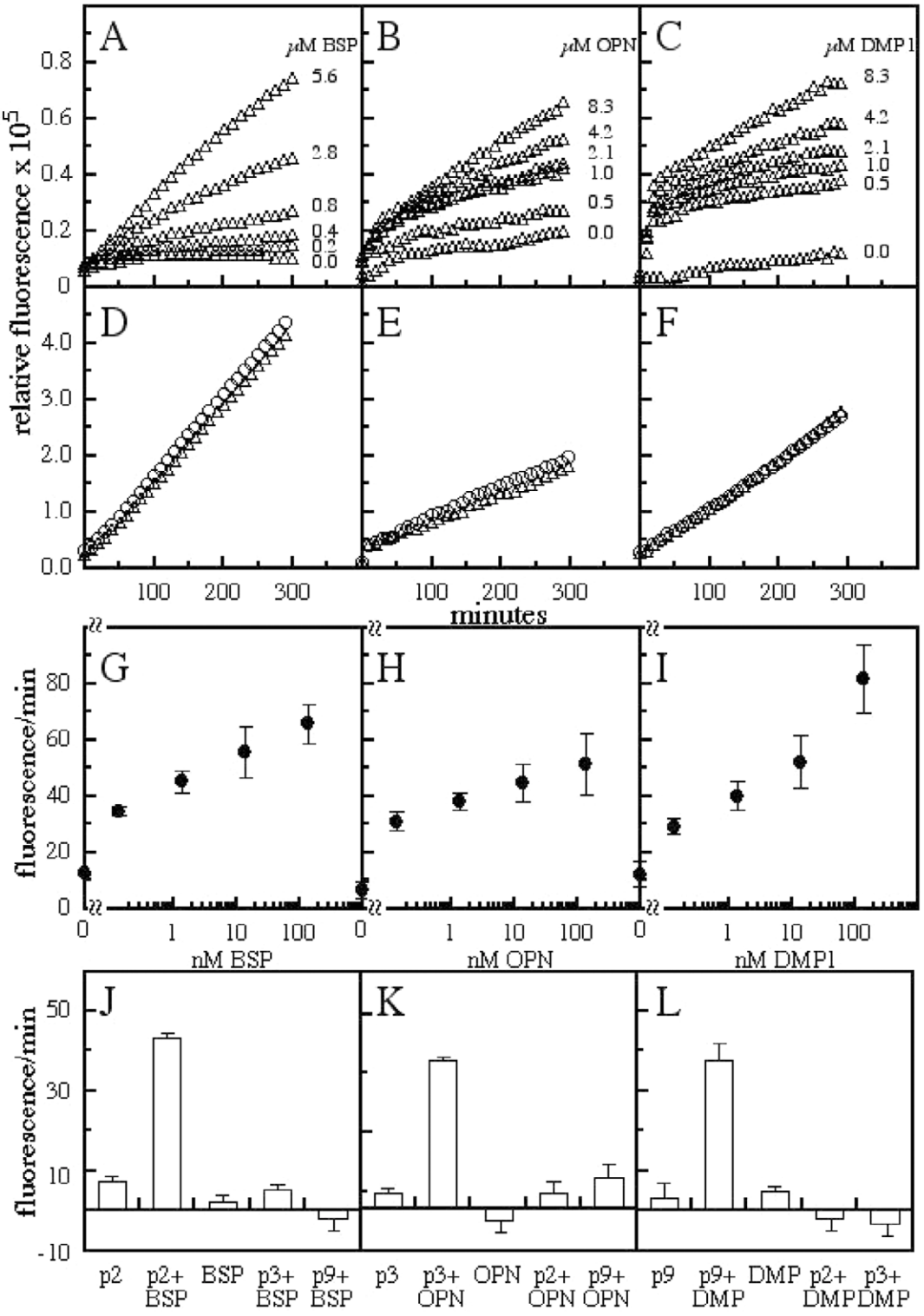

Figure 6. Modulation of MMP activity by SIBLINGs. Protease activity was followed by incubating fluorescent substrate with 1.4 nM proMMP-2 (***A***), proMMP-3 (***B***), and proMMP-9 (***C***) and increasing concentrations of BSP, OPN, or DMP1, respectively. The activity of equimolar concentrations (1.4 nM) of active MMP-2 ± BSP (***D***), MMP-3 ± OPN (***E***), and MMP-9 ± DMP1 (***F***) in the same assay showed no difference. The same assay was also employed to determine a low SIBLING concentration dose–response in activity of proMMP-2 + BSP (***G***), proMMP-3 + OPN (***H***), and proMMP-9 + DMP1 (***I***). The activity of 1.4 nM BSP (***J***), OPN (***K***) and DMP1 (***L***) with correctly matched proMMPs and mismatched proMMPs as well as of proMMPs or SIBLINGs alone were analyzed by determining the change in fluorescence/minute over the first 3 h of incubation. Values plotted represent the mean of three combined experiments ± standard deviation.

Fig. 7

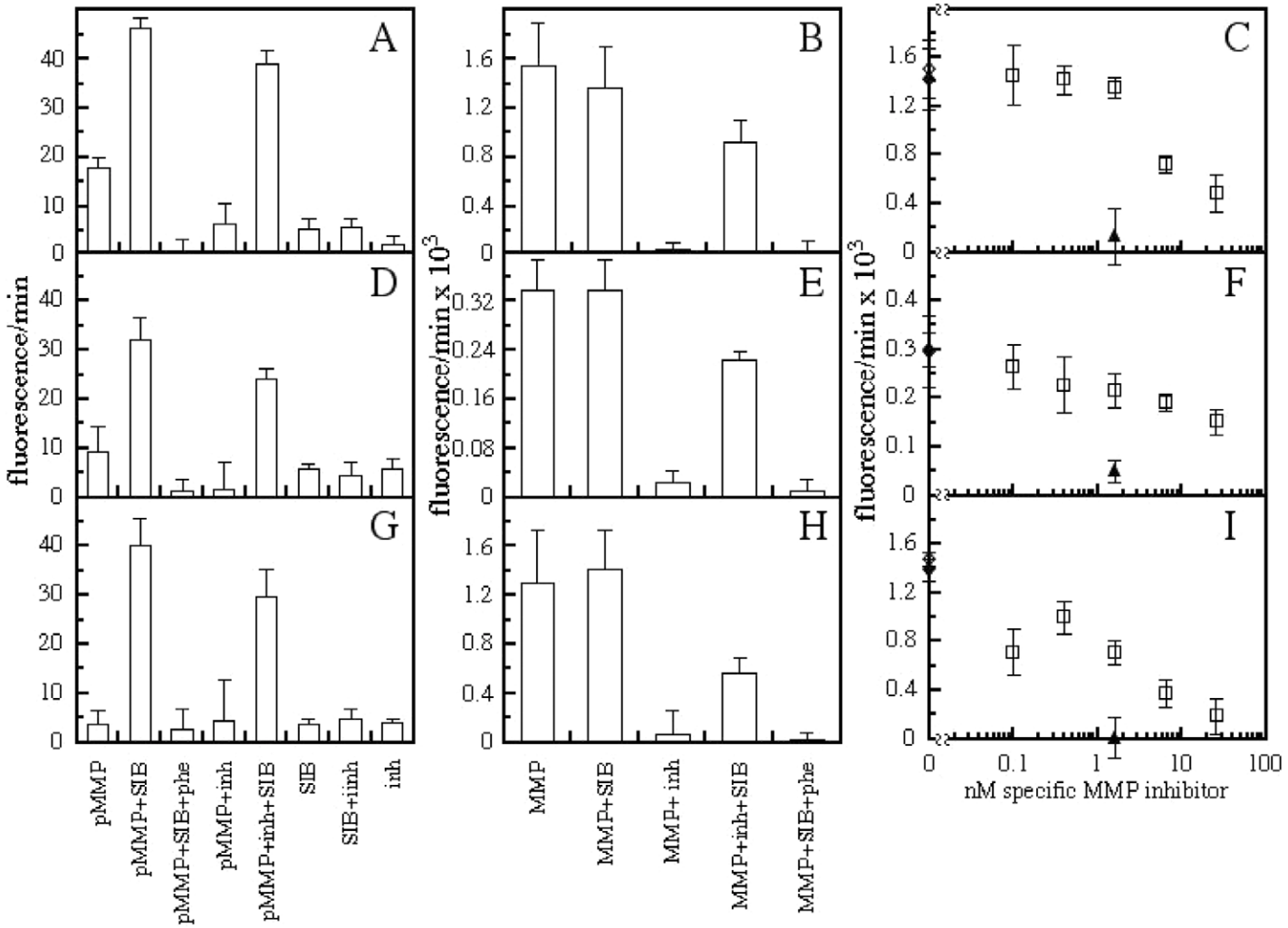

Figure 7. The induced enzymatic activity of MMPs bound with matching SIBLING partners displays altered inhibition by specific low molecular weight inhibitors of MMPs. The ability of the inhibitors to block the SIBLING-activated MMPs was tested using the fluorescein-labeled substrate assay. Substrate was incubated with 1.4 nM MMP alone, MMP + equimolar SIBLING, MMP + equimolar MMP-specific inhibitor, MMP + equimolar MMP-specific inhibitor + equimolar SIBLING, or 1.4 nM MMP + equimolar SIBLING +1,10 phenanthroline. The relative rates of substrate cleavage compared by plotting the average fluorescent change/min are summarized for BSP with proMMP-2 (***A***) or active MMP-2 (***B***), OPN with proMMP-3 (***D***) or active MMP-3 (***E***), and DMP1 with proMMP-9 (***G***) or active MMP-9 (***H***). Values plotted as bars are the mean of four replicates and standard deviations. Abbreviations: pMMP, proMMP; SIB, SIBLING; phe, 1,10 phenanthroline; inh, MMP-specific inhibitor (as described in Materials and Methods). The effect of SIBLING binding was further studied by titration of active MMP + SIBLING complexes with increasing doses of MMP-specific inhibitor for BSP and MMP-2 (***C***), OPN and MMP-3 (***F***), DMP1 and MMP-9 (***I***). For (***C***), (***F***) and (***I***), the symbols represent: open diamond, activity of MMP alone; solid diamond, activity of MMP + SIBLING; solid triangle, activity of MMP + equimolar specific inhibitor; and open square, activity of MMP + SIBLING + specific inhibitor.

Fig. 8

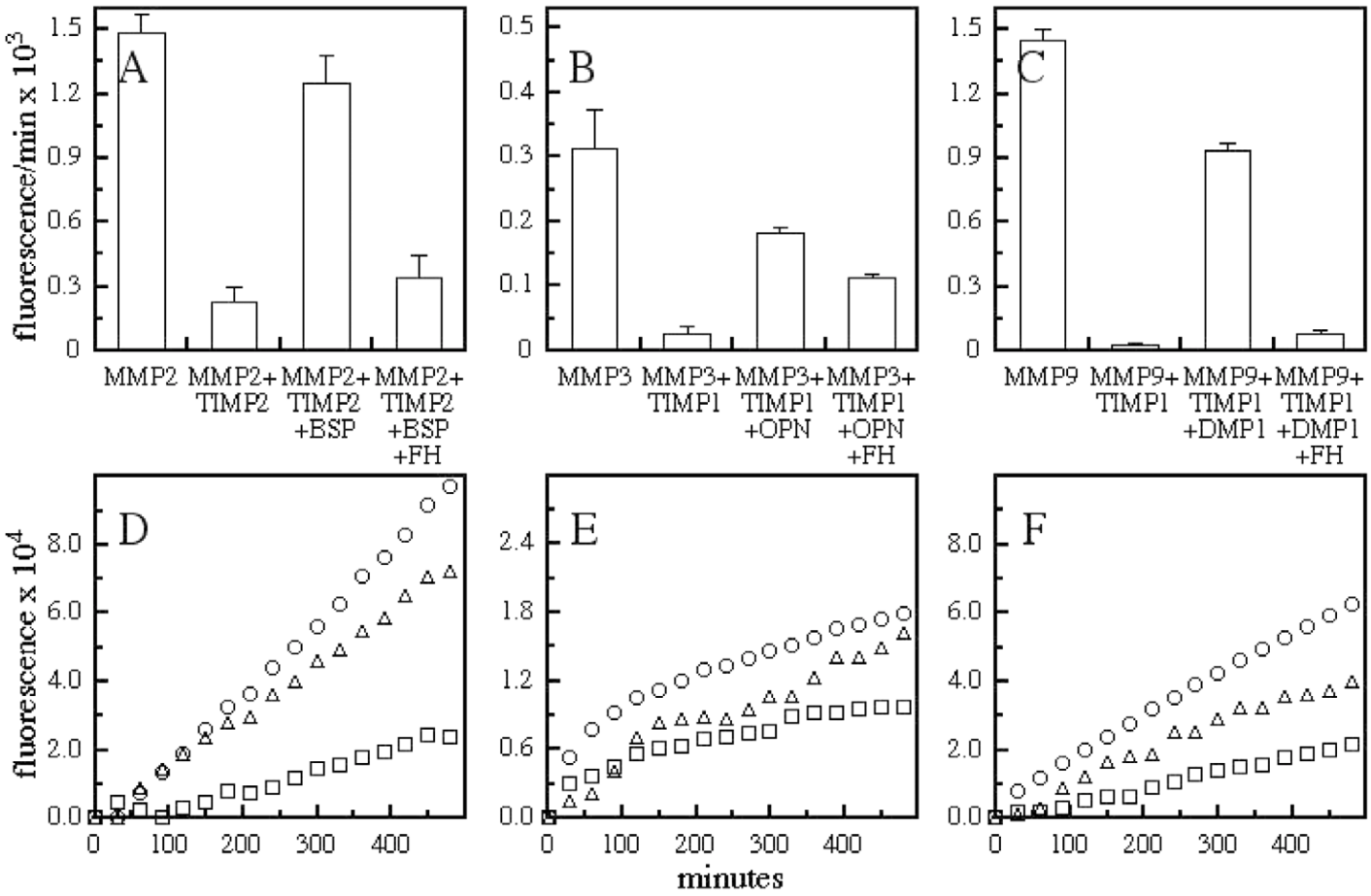

Figure 8. TIMP and Factor H modulation of SIBLING-mediated MMP activation. Active MMP-2 complexed with equimolar TIMP2 and active MMP-3 or MMP-9 complexed with equimolar TIMP1 were incubated for up to 15 h with vehicle, equimolar SIBLINGs, or equimolar SIBLINGs followed by equimolar Factor H (1.4 nM each) in the presence of the fluorescein–gelatin conjugate (***A*** and ***C***) or fluorescein-casein (***B***). The data was analyzed by linear regression analysis over the first 3 h and the slope (fluorescent change/minute) determined. The relative rates of MMP activity were compared by plotting the average of duplicate analyses of fluorescent change/min ± the standard error of the slope for each reaction mixture composition. The ability of Factor H to reverse the SIBLING-mediated activation of proMMPs was investigated using the same assay. 1.4 nM proMMP was incubated with vehicle, equimolar SIBLING, or equimolar SIBLING + Factor H. The equimolar mixtures were BSP and proMMP-2 (***D***), OPN and proMMP-3 (***E***), and DMP1 and proMMP-9 (***F***), where square = proMMP + vehicle; circle = proMMP + SIBLING; and triangle = proMMP + SIBLING + Factor H. Abbreviations: FH, Factor H.